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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Hevesi, et al.	Group Art Unit 1641
Appl. No.	:	09/833,030	
Filed	:	April 10, 2001	
For	:	METHOD FOR OBTAINING A SURFACE ACTIVATION OF A SOLID SUPPORT FOR BUILDING BIOCHIP MICROARRAYS	
Examiner	:	Tran, My-Chau T.	

PP
6/16/05

DECLARATION UNDER 37 C.F.R. §1.132

United States Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Dear Sir:

1. This declaration is being submitted to demonstrate the superiority of the method of the present invention in the production of the microarrays that are highly sensitive, reproducible and stable compared to commercially available solid supports produced by various methods of the prior art.

2. I am an inventor on the above-identified patent application and am familiar with the specification and prosecution history.

3. I have extensive experience in the field of the claimed invention as indicated in the attached Curriculum Vitae provided as Exhibit A of the Declaration submitted Sept. 9, 2002.

4. The Inventor respectfully presents additional examples to demonstrate that using the methodology available at the time the invention was made it was not possible to make a

microarray having a density of DNA molecules fixed to a solid support higher than 220 fmoles/cm² for specific microarray use.

5. The first experiment presents the amount of DNA immobilized on glass submitted to different surface chemistries including amine, carboxylic acid and aldehyde functions added to the solid support surface. The aldehyde slides are commercial slides (Telechem) which were available at the moment of the invention but which were not made by oxidizing olefinic groups present on their surface as claimed in the present patent application. The experiment was performed in optimal coupling conditions for the different reactions which are not directly transposable to the microarray application. Indeed, in this first experiment, 5 µl of DNA was incubated on a small surface of functionalized glass of 25 mm² and incubated for at least 1h at 20°C in a humid chamber to avoid the evaporation of the droplet. Exhibit 1 shows that it is possible to reach a density of 300 fmoles/cm² for the carboxylated glass (condition 4), and about 110 fmoles/cm² for the aldehyde slides (conditions 5 and 6).

6. However, these conditions are not compatible with microarray production for the following reasons. Microarray production has some very specific requirements. The deposit of biological molecules is performed by pins with a very small volume of liquid on the surface (usually in the range of 1 nl compared to the 5 µl used in the experiments of Paragraph 5). Such a small volume evaporates quickly after a deposit at the surface of the solid support (usually in a time interval comprised between 1 and 2 minutes). This means that a reaction cannot be performed for a long time, even if the solid support slides for such spotting are maintained in a humid atmosphere. DNA solutions for such spotting are usually stored in multiple well plates, each well comprising a different DNA solution. The reaction only starts when the DNA solution is in contact with the surface of the solid support. A coupling agent present in the DNA solution sample cannot be maintained stable for a long time and hydrolyses quickly in solution. This means that, once added to the DNA solution, it has to quickly be put in contact with a solid support surface. This is only possible for a first DNA solution to be spotted. For the other solutions there will be a delay time since only one solution can be spotted at a time. Therefore, the use of a coupling agent would introduce high variability in the amount of DNA fixed on the different locations of the formed microarray.

7. The inventors of the above-identified patent application have unexpectedly discovered that the coupling of DNA to a solid support modified with aldehyde functions by oxidizing olefinic groups on the surface of the solid support is certainly the best practical method to make a DNA microarray since it does not require any coupling agent. Therefore, contrary to the use of a coupling agent EDC for the reaction of carboxylic slides, which is not compatible with the reproduction of microarray, amino DNA can be directly bound to a solid support surface comprising aldehyde functions. The process is particularly suited for industrial applications and is actually used as such a commercial product for making DNA chips and protein chips.

8. To reach this goal, a kinetic analysis of reactions was performed with the method of the invention and the method of the state of the art. Exhibit 2 shows that, after 2 minutes incubation, the binding efficiency was about 7% of the value after 4 hours, which corresponds to 14 fmoles/cm². This value is far below the limitation of 220 fmoles/cm² of DNA fixed as recited in the present claim 2.

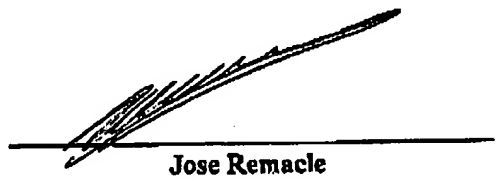
9. The next and last experiment demonstrates the amount of DNA which is effectively fixed in real microarray conditions. The binding efficiency was compared between the aldehyde slide from Telechem and those obtained by the method of the invention (Diaglass). Exhibit 3 shows that the amount of DNA fixed on the Diaglass slide made by the methods claimed in the present application is at least 10 times higher than on Telechem slide. The amount of DNA immobilized on the Telechem slides is lower than 20 fmoles/cm², which is very close to the value obtained in Exhibit for 2 min incubation. Only Diaglass slides made by the methods claimed in the present application allow one to reach a density of 220 fmoles/cm². This clearly shows the advantage of the invention compared to other alternative methods known at the time of the invention for making microarrays.

10. Therefore, at the time this invention was made it was not possible to obtain a microarray on a solid support with a density of at least 220 fmole of DNA molecules/cm² fixed to the surface of the solid support.

11. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief, are believed to be true; and further, these

Appl. No. : 09/833,030
Filed : April 10, 2001

statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under section 1001 of title 18 of the US code and that such willful false statement may jeopardize the validity of the application or patent issuing therefrom.

Dated: 23 March 2005. By: 

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Appl. No. : 09/833,030
Filed : April 10, 2001

Exhibit 1. DNA immobilization on glass using different surface chemistries with long incubation times (not in the conditions of microarray applications)

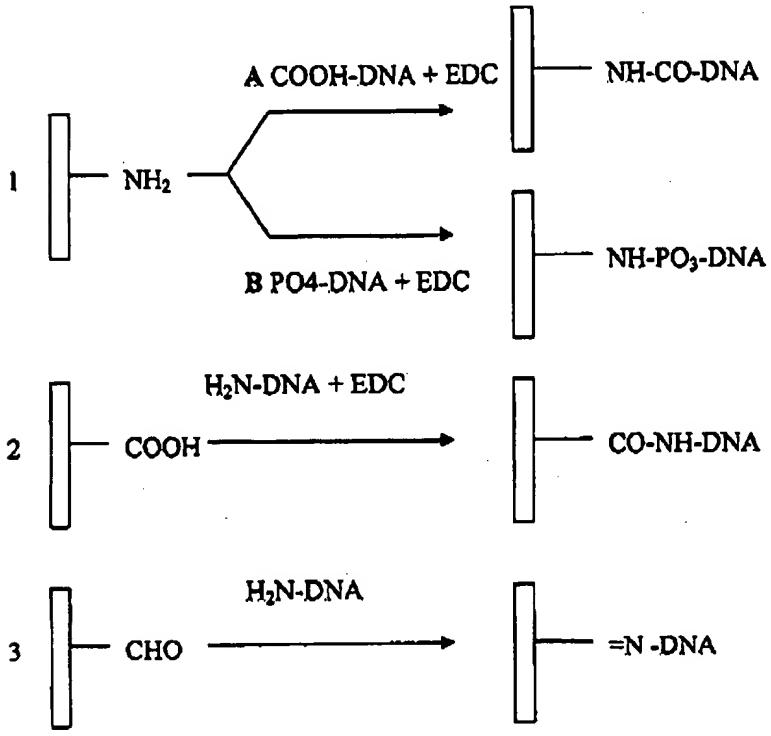
Protocol

Chemicals and buffer

2-(N-morpholino) ethane sulfonic acid (MES) and 1-methylimidazole (MeIm) were from Acros Chimica (Beerse, Belgium). Ethanol, Maleic acid, NaCl, and SDS were from Merck (Darmstadt, Germany). Triethylamine solution, undecenoyl chloride, trifluoroethanol, anhydrous ether, trichlorosilane and hexachloroplatinic acid were from Aldrich chemical (Milwaukee, WI). $NaBH_4$, 1-Ethyl-3-(3 dimethylaminopropyl)-carbodiimide (EDC), Tween 20 and streptavidin-cy3 were from Sigma (St Louis, MO). NHSS was from Pierce (Rockford, IL). Gloria milk powder was from Nestlé (Vervey, Switzerland). [α-^{32}P] dCTP were from Dupont de Nemours (Boston, MA). Oligonucleotides were purchased from Eurogentec (Seraing, Belgium). Untreated glass slides were purchased from Knittel Gläser (Germany). The robot used for microarray fabrication was a Charlyrobot model with 250 µm pins from Genetix (UK). DPX was from BDH Chemicals (UK). The liquid scintillation analyzer LS 60001C was from Beckman Instruments (Fullerton, CA); Aqualuma was from Lumac LSC (Groningen, Netherlands).

Support chemistry

The different surface chemistries compared are summarized as follows.



1. Attachment of carboxylated DNA (a) or phosphorylated DNA (b) to amino-modified glass with EDC.
2. Attachment of aminated DNA to carboxyl-modified glass with EDC.
3. Attachment of aminated DNA to aldehyde-modified glass.

The supports used were: silanated glass-NH_2 (Telechem), synthesized glass-COOH and silylated glass-CHO (Telechem).

Synthesized glass-COOH were prepared using TETU ($Cl_3Si(CH_2)_{10}COOCH_2CF3$). In a 50 ml double-necked flask, 1.12 g (11 mmol) of freshly distilled triethylamine was added to an ice-cooled and stirred solution of 2.02 g (10 mmol) of undecenoyl chloride in 10 ml of anhydrous ether under argon, followed by 1.1 g (11 mmol) of 2,2,2-trifluoroethanol. The mixture was stirred for another 30 min at room temperature, filtered on celite, washed (3x20 ml) with water, dried on magnesium sulfate and evaporated under reduced pressure. 2.5 g (94 %) of TEU (colorless liquid) was obtained.

TETU had been prepared using the following procedure : a mixture of 2.39 g of TEU (9 mmol), 5.42 g de trichlorosilane (40 mmol) and a catalytic amount of hexachloroplatinic acid was stirred under argon atmosphere 2.5 h at room temperature. Excess trichlorosilane was evaporated. The crude product was purified by distillation under reduced pressure (0.2 mbar, 165°C) to obtain 3.07 g (85 %) of TETU (colorless liquid).

Glass slides were cleaned for 30 min in a solution consisting of one-third hydrogen peroxide (30%) and two-thirds sulfuric acid (18 M), rinsed in distilled water, left for 10 min in boiling distilled water, dried under an argon flow and used immediately.

Pre-cleaned microscope slides were immersed for 1 h in a 10^{-3} M solution of TETU in toluene. The samples were then ultrasonically cleaned by three consecutive steps (10 min each) in fresh toluene and dried in an argon flow. The *carboxylic acid terminal groups* were obtained by hydrolysis of ester functionalized slides by immersion into 8 M HCl solution at 95 °C for 2 h. The samples were then ultrasonically cleaned through three consecutive steps (10 min each) in distilled water, dried under an argon flow.

Capture probe synthesis

Cytomegalovirus DNA was used as probe for this study. The capture probes were synthesized by using one modified primer bearing either an amine, a carboxylic acid or a phosphate group at its 5' end. Length of amplicons was 255 bp with one strand bearing at its 5' end the specific chemical function. Radioactive labeling was carried out by the incorporation of [α-^{32}P] dCTP during the PCR amplification. Amplified DNA was separated from unincorporated nucleotides and primers by chromatography on High Pure PCR Product Purification Kit (Boehringer, Mannheim, Germany). DNA concentration was then measured by its absorbance at 260 nm. The purity of the fragment was checked by agarose gel electrophoresis.

DNA immobilization

1. Fixation of phosphorylated DNA to aminated glass (conditions 1 and 2)

A surface of 25 mm² was used in each experiment. Phosphorylated capture probes were denatured for 10 min at 100°C and used at a concentration of 200 nM in 10 mM MeIm pH 7.5 containing 10 mM EDC. 5 µl of the DNA solution were then dispensed onto the 25 mm² aminated glass surface and incubated at 60°C for 5 h in a humid chamber. After incubation, glass samples were washed twice with 5xSSC, 0.25% Tween 20 prewarmed at 50°C and then incubated 3 min with prewarmed water at 95°C to obtain glass bound single stranded capture probes. Quantification of the binding was done by counting the amount of ^{32}P-DNA bound to the glass support with a liquid scintillation counter. Non radioactive samples were stored dried at 4°C.

2. Fixation of aminated DNA to carboxylated glass or carboxylated DNA to aminated glass (conditions 3 and 4)

Aminated or carboxylated capture probes were denatured for 10 min at 100°C and diluted to a concentration of 200 nM in 0.1 M MES buffer pH 6.5 containing 5 mM EDC and 0.33 mM NHSS. 5 µl of DNA solution was dispensed onto carboxylated or aminated 25 mm² glass surface and incubated for 2 h at 20°C in a humid chamber. Washing and denaturation procedures were then performed as described just above.

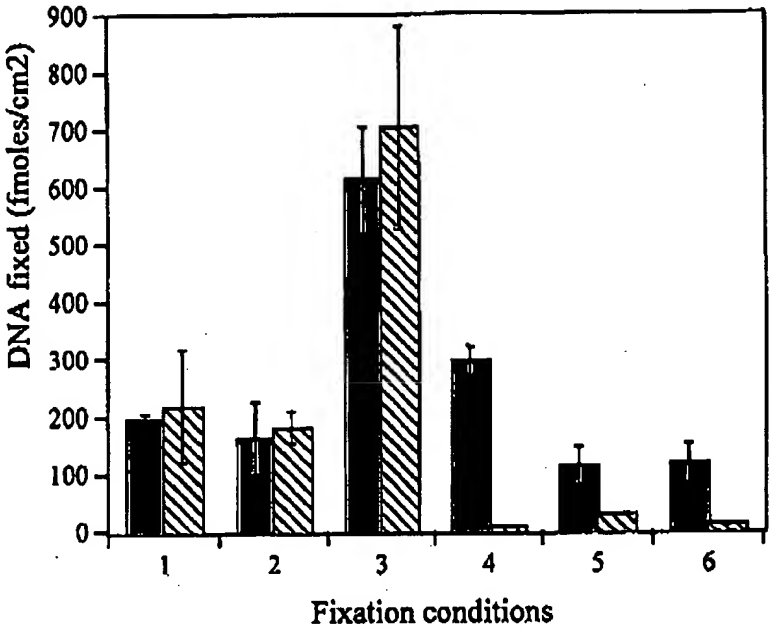
3. Fixation of aminated DNA to aldehyde glass (conditions 5 and 6)

Aminated capture probes were denatured for 10 min at 100°C and diluted to a concentration of 200 nM in 0.1 M MES buffer pH 6.5. 5 µl of DNA solution was dispensed onto the aldehyde glass surface and incubated for 1 h at 20°C in a humid chamber.

The different DNA probes labelled with ^{32}P and incubated with different derivatized surfaces at a concentration of 200 nM. For carbodiimide mediated bindings, background was determined by carrying out the reaction in the absence of EDC (EDC-) while in the aldehyde assay, phosphorylated DNA was used as negative control. All reactions were performed in optimal conditions and in a humid chamber to maintain the DNA in solution.

Appl. No. : 09/833,030
Filed : April 10, 2001

Results



DNA immobilization on glass using different surface chemistries.

Legend : fixation conditions (bars 1-6) : (1) Telechem glass-NH_2 batch 1, PO_4-DNA; (2) Telechem glass-NH_2 batch 2, PO_4-DNA; (3) Telechem glass-NH_2, COOH-DNA; (4) Synthesized glass-COOH, NH_2-DNA; (5) Telechem glass-CHO batch 1, NH_2-DNA; (6) Telechem glass-CHO batch 2, NH_2-DNA. Fixations were obtained with (black) or without EDC (hatching) in conditions 1-4 and with aminated DNA (black) or phosphorylated DNA (hatching) in conditions 5-6. Results were expressed as the amount of ^{32}P capture probe DNA fixed per 25 mm^2 sample. They are means ± 2 SD of three values. Quantification was performed by liquid scintillation counting.

Quantitative data:

Conditions	Test (black)	Control (hatching)	SD Test	SD Control
1	196	216	5	98
2	160	179	63	26
3	610	702	93	177
4	295	7	26	1
5	110	28	34	1.5
6	116	10	34	1.3

Discussion

DNA coupling with long incubation time was obtained for all strategies, but with different yields. On aminated glass (lanes 1-3), fixation of DNA was very good especially with carboxylated DNA since 700 fmoles of probes could be fixed per cm^2 (lane 3). However, in this case, the non specific binding (without EDC) was as high as the test. This is probably due to the electrostatic attraction between the negatively charged DNA and the positive surface.

On neutral (aldehyde functions) or negative (carboxylic functions), such non specific bindings were not observed. Indeed, DNA probes could be fixed specifically either on the aldehyde (lane 5-6) or even better on the carboxylated surface (lane 4). Similar yield was obtained with the aldehyde commercial (lane 5) or synthesized slides (lane 6). The density of DNA fixed was around 110 fmoles/cm^2 on the aldehyde slides after 1h incubation at 20°C and around 300 fmoles/cm^2 on the carboxylated slides after 2h incubation at 20°C.

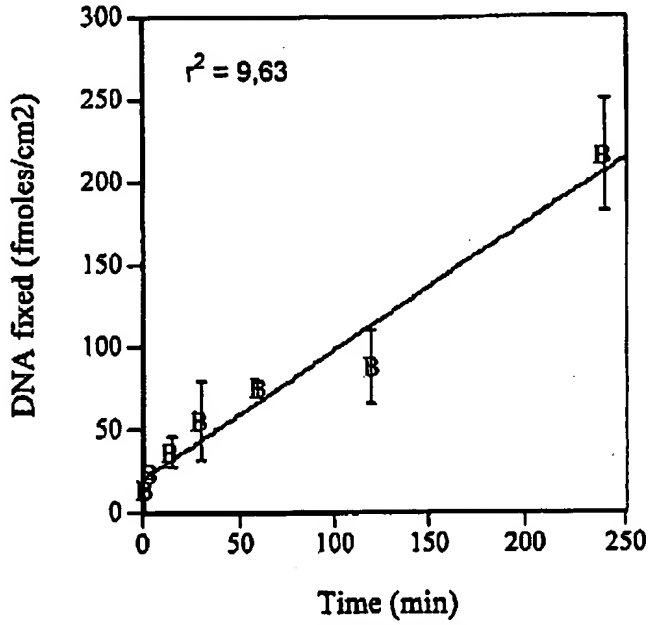
Appl. No. : 09/833,030
Filed : April 10, 2001

Exhibit 2. Capture probe immobilization kinetics on Telechem aldehyde slides

Protocol

The experiment was performed as described in the protocol of Exhibit 1 for the binding of aminated DNA on Telechem aldehyde slides. DNA binding kinetics was evaluated by varying the fixation time of 5 µl of capture probes at a concentration of 200 nM.

Results



Capture probe immobilization kinetics on Telechem aldehyde slides.
Legend : five µl of aminated capture probe solution at 200 nM were applied to the CHO-derivatized glass surface from Telechem. The coupling time varied from 2 min to 240 min at room temperature. Results are expressed as the amount of ^{32}P capture probe DNA fixed. They are means ± 2 SD of three values. Quantification was performed by liquid scintillation counting.

Quantitative data:

Time (min)	DNA fixed (fmoles/cm²)	Standard deviation
2	14	1
5	23	2
15	36	9
30	55	24
60	74	5
120	87	22
240	215	34

Discussion

In the conditions used, there was no significant probe evaporation. A linear relationship was obtained between the surface density and the time of contact. After 2 min incubation, the binding efficiency was about 7 % of the value after 4 h and corresponds to 14 fmoles/cm².

Appl. No. : 09/833,030
Filed : April 10, 2001

Exhibit 3. Capture probe density on Diaglass and Telechem aldehyde slides in microarray conditions

Protocol

Fixation of capture probes to aldehyde glass using an arrayer

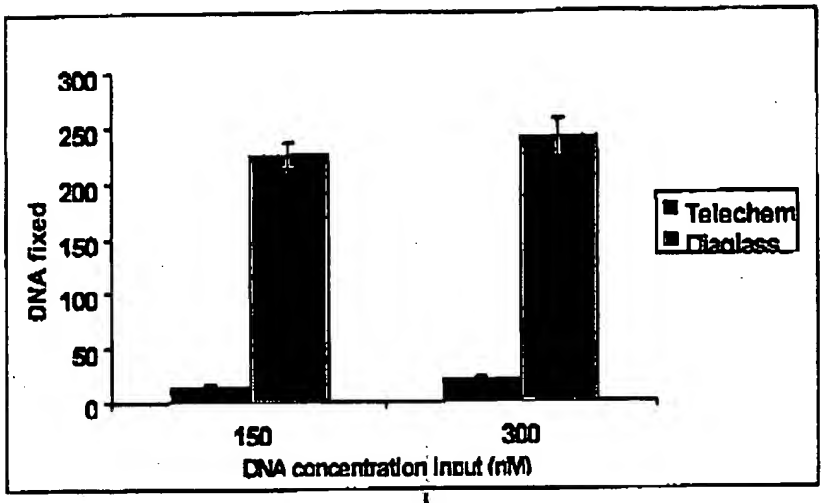
Capture probes were synthesized as described in the protocol of figure 1. P^{32}-labelled and aminated capture probes are diluted to a concentration of 300 nM and 150 nM in either SSC3X buffer pH 5, 0.01% SDS or SSC3X buffer pH 5. Final volume is 25 µl. Solutions with SDS are used for the spotting on DIAGLASS slides made according to the invention and those without SDS on aldehyde slides from Telechem. Probes are dispensed on the glass slides with an arrayer. Each array is composed of 100 spots (10 X10). The spots are 400 µm in diameter and distance between two adjacent spots is 500 µm. After 1 h incubation at 23°C, glass samples are washed once with 0.1% SDS, twice with water, then incubated for 5 min with sodium borohydride solution (2.5 mg $NaBH_4$ dissolved in 750 µl of PBS and 250 µl of 100% ethanol), one with water and finally 3 min with boiling water to obtain single stranded probes on the surface.

Controls arrays (100% fixation) do not include this washing step. Glass samples including the array are cut and quantification of the binding is done by counting the amount of ^{32}P-DNA bound to the glass support with a liquid scintillation counter.

Calculation of the capture probe density

The amount (in moles) of bound DNA in each array is calculated in reference to the 100% fixation controls. This value is then divided by a factor of 100 to obtain the fixed amount in each spot. Knowing the spot diameter (400 µm), the spot surface can be calculated and is equal to 125000 μm^2. Finally, the amount of DNA fixed per cm^2 can be deduced.

Results



Influence of the support used (Diaglass or Telechem slides) on the capture probe density.
Legend : radiolabeled capture probes of 257 bp are fixed on Diaglass or Telechem slides at concentrations of 150 and 300 nM. After washing, the amount of single stranded probe covalently fixed is calculated. Results show a ten times increase in the amount of DNA probe fixed onto Diaglass slides. This can be explained by a higher aldehyde density on Diaglass slides allowing the fixation of an increased amount of capture probe.

Discussion

The amount of DNA fixed on the Diaglass slides is at least 10 times higher than on the Telechem slide. The amount of DNA immobilized is lower than 20 fmoles/cm² which is very close to value obtained in Figure 2 for 2 min incubation. Only the Diaglass slides allow to reach a density of 220 fmoles/cm². The DNA concentration in the input (150 or 300 nM) does not affect significantly the binding yield.